

501262

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
17 July 2003 (17.07.2003)

PCT

(10) International Publication Number
WO 03/057915 A2

- (51) International Patent Classification⁷: **C12Q 1/68**
- (21) International Application Number: PCT/GB03/00064
- (22) International Filing Date: 10 January 2003 (10.01.2003)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
0200526.2 11 January 2002 (11.01.2002) GB
- (71) Applicant (*for all designated States except US*): **UNIVERSITY OF SOUTHAMPTON** [GB/GB]; Centre for Enterprise and Innovation, Highfield, Southampton SO17 1BJ (GB).
- (72) Inventor; and
- (75) Inventor/Applicant (*for US only*): **ROSENBERG, William, Malcolm, Charles** [GB/GB]; Centre for Enterprise and Innovation, Highfield, Southampton SO17 1BJ (GB).
- (74) Agents: **LEE, Nicholas, John** et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— *without international search report and to be republished upon receipt of that report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: METHODS

(57) Abstract: The present invention provides a method of detecting HCV genotype 1 (HCV-1) in a sample which is based on the finding that the 5' non-coding region (NCR) of the HCV genome is conserved between HCV-1 quasi-species but not between other HCV subgroups. The method comprises subjecting the sample to an amplification reaction using at least one primer which anneals specifically to the 5' noncoding region (5' NCR) of the HCV-1 genome, and detecting the product of the amplification reaction. Kits and primers suitable for carrying out the method are also provided.

BEST AVAILABLE COPY

METHODS

This invention relates to methods for detecting viral genotypes, and in particular Hepatitis C virus (HCV) genotypes. The invention therefore finds application in determining prognosis, and in the selection of treatment regimes, for patients infected with HCV.

The hepatitis C virus is a positively stranded RNA virus that exists in six genetically distinct genotypes. These are designated Type 1, 2, 3, 4, 5 and 6, and their full length genomes have been reported (Genbank/EMBL accession numbers Type 1a: M62321 (Choo), AF009606, AF011753, Type 1b: AF054250, D13558, L38318, U45476, D85516; Type 2b: D10988; Type 2c: D50409; Type 3a: AF046866; Type 3b: D49374; Type 4: WC-G6, WC-G11, WG29 (Li-Zhe Xu *et al*, *J.Gen. Virol.* 1994, 75:2393-98), EG-21, EG-29, EG-33 (Simmonds *et al*, *J. Gen. Virol.* 1994, 74: 661-668)). Viruses in each genotype exist as differing "quasispecies" that exhibit minor genetic differences. In the UK and USA, the vast majority of infected individuals are infected with genotype 1, 2 or 3 HCV. In the UK, the only other genotype encountered is genotype 4 which is identified at low prevalence in some immigrant populations. HCV infection affects approximately 0.5% of the population in the United Kingdom, 1.8% of the population in the USA and 3% of the population of the world. In over 85% of infected people, HCV causes a lifelong infection characterised by chronic hepatitis that varies in severity between individuals.

The only effective therapy that is currently available is interferon alpha (IFN- α) used alone or in conjunction with ribavirin ("combination therapy"). Randomised controlled trials have shown that combination therapy is highly effective in the treatment of HCV infection in patients previously treated with interferon alone and in patients never previously treated with interferon (Davis *et al*, *NEJM*, (1998), 339(21): 1493-99; Poynard *et al*, *Lancet* (1998) 352(9138): 1426-32).

Subgroup analysis has revealed that the major determinant of prognosis and response to treatment is HCV genotype (Poynard *et al*, *Lancet*, 1998, 352(9138): 1426-32). For patients with genotype 2 or 3 infections treated for six months, response rates to treatment for six months are equivalent to those seen in patients with genotype 1 infections for one year. There were insufficient data in the trials on response rates for patients with genotype 4, 5 and 6 infections to permit investigators to determine response rates in patients with these infections. In mixed genotype infections, genotype 1 predominates.

Combination therapy is expensive (in the UK, it costs approximately £8,000 for one year's treatment). Thus, the identification of patients with non-genotype 1 infections allows treatment to be stopped after 6 months with a consequent cost saving, and the avoidance of unnecessary adverse effects of the drugs associated with further treatment. In addition, the most serious outcome of HCV infection is hepatocellular carcinoma. It is therefore desirable to be able to identify with which genotype(s) of HCV a patient is infected.

Known HCV genotyping methods rely on agarose gel electrophoresis of restriction endonuclease digested polymerase chain reaction amplification products (RFLP) or antibody detection of anti-HCV antibodies. Enzymatic digestion can take a relatively long time to reach completion making the RFLP method time consuming. It also involves several different stages, making it unamenable to automation or rapid throughput. In addition, inaccurate genotyping can occur owing to (i) the fact that restriction endonucleases do not cut with 100% efficiency, and (ii) single base pair mutations in quasispecies may result in failure of digestion of the PCR products. Antibody based methods are costly and relatively insensitive. Most significantly, they are incapable of differentiating between patients who have cleared HCV infection and those with persisting infection. They thus require the performance of a virological test in parallel to confirm current infection.

US Patent No. 5851759 discloses methods of genotyping HCV in which HCV RNA is isolated and cDNA is synthesised from this RNA. The cDNA is then subjected to PCR using primers which flank the E1 region of the HCV genome. The products of PCR are analysed using the Heteroduplex Tracing Assay (HTA). The E1 region was selected for analysis because it is considered to be the most heterogeneous region of the HCV genome. Thus, amplification of this region is likely to give a different product for each HCV strain in a sample. These products can then be analysed to indicate the strains that are present in the sample.

In view of the different effect of combination therapy on infection with HCV 1 as compared to infection with other HCV subtypes, there exists a need to be able to detect HCV genotype 1.

According to the present invention, there is provided a method of detecting HCV genotype 1 (HCV-1) in a sample, comprising:

subjecting the sample to an amplification reaction using at least one primer which anneals specifically to the 5' noncoding region (5' NCR) of the HCV-1 genome; and

detecting the product of the amplification reaction.

The HCV genome consists of 5' and 3' noncoding (or untranslated) regions (NCR) that flank a single long open reading frame (ORF). This ORF encodes for three structural proteins at the amino-terminal end and for six non-structural (NS) proteins at the carboxyl-terminal end. The structural proteins are represented from the nucleocapsid (core; C) proteins and two glycoproteins, envelope 1 (E1) and envelope 2 (E2). The non-structural proteins are named NS2, P7, NS3, NS4a, NS4b, NS5a, NS5b. The 5' NCR is the most highly conserved part of the HCV genome, whereas the sequence of the two envelope proteins (E1 and E2) is highly variable among different HCV isolates. The present invention lies in the surprising finding that parts of the 5' NCR

are conserved between HCV-1 quasi species but not between other genotypes. A preferred region is the region between residues -134 and -118 of the 5'NCR of HCV-1. Accordingly, primers for amplification reactions can be designed which anneal specifically to those parts of the 5' NCR that are conserved between HCV-1 quasi species but not between other genotypes.

The amplification reaction may be PCR (see for example U.S. Patents Nos. 4,683,195 and 4,683,202, and Innis *et al*, editors, PCR Protocols, (Academic Press, New York, 1989; Sambrook *et al*, Molecular Cloning, Second Edition, (Cold Spring Harbour Laboratory, New York 1989)). PCR will generally be used when HCV RNA has been isolated and converted, preferably by reverse transcription, to cDNA. Preferably, PCR is carried out using Taq DNA polymerase, e.g. Amplitaq™ (Perkin-Elmer, Norwalk, Conn.). Taq polymerase can also be obtained from MBI Fermentas, Perkin Elmer, Boehringer Mannheim and Beckman Instruments. An equivalent, preferably thermostable, DNA polymerase may also be used in the method of the present invention, such as Tfl (*Thermus flavus*) polymerase (Gut *et al*, *Viol. Methods* 77(1): 37-46 (1999)).

Alternatively, the amplification reaction may be RT-PCR (Yajima *et al*, *Clin. Chem*, 44(12): 2441-2445 (1998); Martell *et al*, *J. Clin. Microbiol.*, 37(2): 327-332 (1999); Gut *et al*, *Viol. Methods* 77(1): 37-46 (1999); Predhomme *et al*, *Leukemia*, 13(6): 957-964 (1999)), in which the HCV RNA is reverse transcribed into cDNA which is then subjected to PCR amplification.

As is well-known, PCR involves the extraction and denaturation (preferably by heat) of a sample of DNA (or RNA). A molar excess of oligonucleotide primers is added, along with a polymerase, which may be heat-stable, and dNTPs for forming the amplified sequence. The oligonucleotide primers are designed to hybridise to opposite ends of the sequence which it is desired be amplified. In the first amplification round, the polymerase replicates the DNA to produce two "long products," which begin with

the respective primers. The total DNA, which includes the two long products and the two original strands, is then denatured and a second round of polymerisation is carried out (for example, by lowering the temperature). The result of the second round is the two original strands, the two long products from the first round, two new long products (produced from the original strands), and two "short products" produced from the long products. These short products have the sequence of the target sequence (sense or antisense) with a primer at each end. For each additional amplification round, the number of short products grows exponentially, each round producing two additional long products and a number of short products equal to the sum of the long and short products remaining at the end of the previous round.

Oligonucleotide primers can be synthesised by a number of approaches, e.g. Ozaki *et al*, *Nuc. Acids Res.* 20: 5205-5214 (1992); Agrawal *et al*, *Nuc. Acids Res.* 18: 5419-5423 (1990) or the like. Conveniently, the oligonucleotide probes are synthesised on an automated DNA synthesiser, e.g. an Applied Biosystems, Inc, Foster City, California model 392 or 394 DNA/RNA synthesiser using standard chemistries such as phosphoramidite chemistry (Beaucage and Iyer, *Tetrahedron* 48: 2223-2311 (1992), US Patent Nos. 4980460, 4725677, 4415732, 4458066 and 4973679). Alternative chemistries, including non-natural backbone groups such as phosphorothioate and phosphoramidate, may also be employed, provided that the hybridisation efficiencies of the resulting oligonucleotides are not adversely affected. The precise length and sequence of the DNA primers will depend on the target polynucleotide to be amplified. Preferably, the length of the DNA primers is in the range 10 to 60 nucleotides and more preferably in the range 15 to 30 or 25 nucleotides.

In a preferred embodiment, the method of the present invention further comprises a step in which HCV infection generally is detected. In this step, the sample is subjected to an amplification reaction using primers which anneal to a region of the 5'NCR which is conserved between all HCV genotypes and the product of this amplification reaction is detected. Preferably, the amplified region spans the region

which is conserved in HCV-1. This additional step allows verification that HCV is present in the sample and so treatment can be tailored accordingly if no HCV-1 is detected.

- 5 Detection of the products of amplification may be carried out using the well-known technique of agarose gel electrophoresis. If HCV-1 is present in the sample, the amplification reaction produces a product (of known size) which can be detected on the agarose gel. An HCV-1 specific primer suitable for this is the DNA oligonucleotide:

10

5' CCI CTC AAT GCC TGG AG 3' (*Spec-1*)

Spec-1 may be used together with any universal HCV primer. One example is:

- 15 Reverse: 5' GCA GTA CCA CAA GGC CTT TCG C 3' (*UTR-R2*)

The invention provides such primers alone and in combination.

- 20 If it is desired to carry out an amplification reaction additionally to detect whether HCV in general is present in the sample, a pair of primers universal for all HCV genotypes can be used. This will produce a product of a known size which is different to that for the HCV-1-specific amplification. If HCV-1 and other HCV genotypes are present, the gel will show both products. Suitable primers for this are:

- 25 Forward: 5' CGT CTA GCC ATG GCG TTA G 3' (*UTR-L2*)
Reverse: (*UTR-R2*)

- 30 Additionally, a preliminary amplification may be carried out on the sample to isolate HCV material. This preliminary amplification can be carried out using two primers universal for all HCV genotypes. Suitable primers for this are:

Forward: 5' GGA ACT ACT GTC TTC ACG C 3' (*UTR-L1*)

Reverse: 5' ACG GTC TAC GAG ACC TC 3' (*UTR-R1*)

5 Detection of the products of amplification may alternatively be carried out using
fluorescent analysis. If genotype 1 HCV is present, amplification occurs and this is
detected by hybridisation of a quenched fluorescently labelled probe. With successive
rounds of amplification, an increasing amount of the fluorescent probe becomes
incorporated into PCR products and the quencher is liberated resulting in an increasing
10 amount of fluorescence being detectable on the reaction vessels. Such fluorescence
detection makes this approach ideally suited to automation and high throughput
processes.

One well-known fluorescent technique is the so-called "TaqMan" method (Holland, *et*
15 *al*, *Proc. Nat. Acad. Sci*, 88: 7276-80, (1985)). This method uses an oligonucleotide
probe complementary to a segment of the target polynucleotide, i.e. between the
sequence defined by the PCR primers, which probe is labelled with two fluorescent
molecules or fluorophores. The emission spectrum of one of the molecules overlaps
with the excitation spectrum of the other and, as a result, the emission of the first
20 fluorophore (the "reporter") is quenched by the second (the "quencher") when they are
in sufficient proximity.

The oligonucleotide probe is hybridised to the target polynucleotide downstream from
a primer for a polymerase having a 5'-3' exonuclease activity. As the polymerase
25 extends the primer as part of the PCR reaction, the oligonucleotide is digested,
releasing one of the "quencher" and "reporter" molecules and causing the distance
between these molecules to become such that the fluorescent emission is no longer
quenched by energy transfer. Thus, a fluorescent signal is generated, providing real-
time monitoring of amplification.

The oligonucleotide probe may be made in the same way as the oligonucleotide primers mentioned above and may have the same variations in backbone and so on, providing that hybridisation to the target polynucleotide is not compromised and/or cleavage efficiency of the exonuclease are not adversely affected. Preferably, the length of the oligonucleotide probe is in the range of 10 to 60 nucleotides, and more preferably 18 to 30 nucleotides. The precise length and sequence of the oligonucleotide probe depends at least in part on the nature of the target polynucleotide to which it binds and will be varied to achieve appropriate annealing and melting properties for a particular target polynucleotide. In addition, the binding location of the probe may be varied according to the nature of the target polynucleotide.

Preferred primers for this reaction are *UTR-R2* and *Spec1* mentioned above. A preferred probe has the following sequence and is specific to HCV-1:

5' FCG CIA CCC AAC ICT ACT IGG CTA GT 3' (*L1*)

where F=6-FAM, 3'-T+TAMRA

Alternatively, the technique such as the TaqMan technique can be used to detect HCV-1 using one or more universal primers and an HCV-1 specific probe. Thus, in a further aspect, the present invention provides a method of detecting HCV genotype 1 (HCV-1) in a sample, comprising:

subjecting the sample to an amplification reaction using at least one primer which anneals to the genome of HCV, a polymerase having a 5'-3' exonuclease activity and an oligonucleotide probe, which probe anneals specifically to the 5' noncoding region (5' NCR) of the HCV-1 genome and which incorporates a modified nucleotide having a fluorescent characteristic which is modified by one or more neighbouring nucleotides; and

detecting a change in fluorescence as the oligonucleotide probe is degraded by the exonuclease activity of the polymerase as the polymerase extends the primer and modification of the fluorescent characteristic of the modified nucleotide is reduced.

5 The modified nucleotide is preferably a "reporter" molecule and the neighbouring nucleotide(s) is/are preferably a "quencher" molecule. The primer(s) may be universal and is/are preferably *UTR-L1* and *UTR-R1* or *UTR-L2* and *UTR-R2*. Alternatively, the probe(s) may be capable of annealing specifically to the 5' NCR of the HCV-1 genome. The probe may be *L1* as defined above.

10

A further alternative method of detecting the amplification products is using one or more molecular beacon probes. These are PCR primers comprising a sequence attached to a hairpin loop sequence which contains both a fluorescent label and a quencher molecule. The hairpin brings the fluorescent molecule in opposition to the quencher so that the primer does not fluoresce under normal conditions. As PCR results in copying of the probe sequence, the hair-pin opens and the fluorescent beacon and quencher become separated. The resulting PCR product fluoresces.

15

In a preferred embodiment, the following PCR primers are used:

20

Forward: *MBP-LR-1*

5'FCA CCT TCA CCC TCA GAA GGM GCC GCT CAA TGC CTG GAG3'

(F=FAM; M=MeREDdU and U=Uracil)

25

Reverse: *UTR-R2*

MBP-LR-1 is a forward HCV type 1 specific primer and, as mentioned above, *UTR-R2* is a universal reverse primer. Any universal HCV primer can be used together with *MBP-LR-1*.

30

If it is desired to detect the presence of other HCV genotypes in the sample, an additional amplification can be carried out using at least one molecular beacon probe which is universal for all HCV genotypes. A suitable probe in this regard is *MBP-LR-ALL* which is a forward primer and has the following sequence:

5

5'FCACCTTCACCCTCAGAAGGMGCGUCTAGCCATGGCGTTAG3'

(F=FAM; M=MeREDdU and U=Uracil)

10 *MBP-LR-ALL* can be used together with any universal reverse primer; one suitable example is *UTR-R2*.

Fluorochromes that emit light at differing wavelengths can be incorporated into the different primers so that products amplified with different primers can be distinguished in the same reaction vessel. Alternative hairpin sequences on the beacon probes can easily be designed by the skilled person.

15

Alternatively, the amplification products can be detected using DNA hybridisation employing enzyme linked methods to confirm hybridisation, such as using horse radish peroxidase on sequence specific probes and an appropriate substrate.

20

According to a further aspect of the present invention, there is provided a kit for detecting HCV genotype 1 (HCV-1) in a sample, comprising: at least one primer which anneals specifically to the 5' noncoding region (5' NCR) of the HCV-1 genome.

25

The kit may further comprise the polymerase and an appropriate primer or set of primers. In addition, it may comprise additional reagents that are necessary for performing the invention, such as dNTP mixtures, buffers, molecular size standards, wax beads and the like. The reagents may be provided in pre-measured amounts so as to simplify the

performance of the method. Typically, the kit may also contain detailed instructions for carrying out the method.

5 It will be appreciated by those skilled in the art that the sequences of the primers and probes described above can be modified without affecting their activity, i.e. their ability to act as primers or probes in the methods of the present invention. Such modified probes and primers are included in the present invention, provided that they have substantial identity with the probes mentioned above.

10 When comparing nucleic acid sequences for the purposes of determining the degree of homology or identity, one can use programs such as BESTFIT and GAP (both from the Wisconsin Genetics Computer Group (GCG) software package). BESTFIT, for example, compares two sequences and produces an optimal alignment of the most similar segments. GAP enables sequences to be aligned along their whole length and finds the
15 optimal alignment by inserting spaces in either sequence as appropriate. Suitably, in the context of the present invention when discussing identity of nucleic acid sequences, the comparison is made by alignment of the sequences along their whole length.

20 Preferably, sequences which have substantial identity have at least 75% sequence identity and more preferably at least 90 or at least 95% sequence identity with said sequences. In some cases, the sequence identity may be 99% or above.

25 Desirably, the term "substantial identity" indicates that said sequence has a greater degree of identity with any of the sequences described herein than with prior art nucleic acid sequences.

30 Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law. The abbreviations for nucleotides used herein are standard unless otherwise indicated.

The present inventors have invented a polymerase chain reaction amplification (PCR) based test that permits the detection of all types of HCV infection and the specific identification of HCV genotype 1 infections.

5

The present invention will now be described further in the following examples. Reference is made to the accompanying drawings:

10 Figures 1a and 1b show 2% agarose gels of PCR products stained with ethidium bromide to show bands 88 base pairs in size (genotype 1 specific band) and 217 base pairs (all HCV genotypes).

15 Figures 2a-f show printouts of PCR runs using fluorescent probe detection and Taqman technology. Fluorescence intensity is represented on the y axis and the number of PCR cycles elapsed is shown on the x axis.

20 Figures 3a and 3b are printouts of PCR runs using fluorescent probe detection and Taqman technology. Fluorescence intensity is represented on the y axis and the number of PCR cycles elapsed is shown on the x axis.

20

Figure 4 shows printouts of PCR runs using molecular beacon probe analysis and fluorescence detection. Fluorescence intensity is represented on the y axis and the number of PCR cycles elapsed is shown on the x axis.

25 Figure 5 is a diagram illustrating the positions of the various primers and probes used in certain embodiments of the present invention within the 5'NCR of the HCV-1 genome.

Examples

Example 1

5 From a 200µl sample of serum, HCV RNA was extracted using mRNA extraction kit (QIAGEN Ltd, Crawley, West Sussex) as per manufacturer's instructions. 11.5µl of RNA solution as extracted above was added to 0.5 µl of random primers (Promega UK Ltd, Southampton) in a 1.5 µl eppendorf test tube and incubated at 90 °C for 5 minutes in a heating block and then snap cooled on ice.

10

cDNA was then prepared by reverse transcription using the following reaction mix:

5 µl of 5x SuperScript buffer (Life Technologies (GIBCO BRL) Paisley, Scotland);
2.5 µl of 100 mM DTT; 2.5 µl of 1mg/ml Bovine Serum Albumin acetylated (10
15 mg/ml) (Promega, UK Ltd, Southampton); 1.25 µl of 10mM dNTP solution (Amersham Pharmacia, Buckinghamshire); 1.25 µl Superscript Moloney Murine Leukaemia Virus reverse transcriptase (Life Technologies (GIBCO BRL) Paisley, Scotland); 0.5 µl of RNasin ribonuclease inhibitor (Promega, UK Ltd, Southampton).

20 13 µl of this solution was added to each tube containing RNA/random primers. The mix was incubated at 42 °C for 60 minutes. The reaction was stopped by heating to 95 °C for 5 minutes. The cDNA was then stored at -20 °C.

1µl of the resulting complimentary DNA (cDNA) was suspended in 20µl of a first
25 round reaction mix. This mix contains 1.5mM MgCl₂, 0.2mM deoxy nucleotide triphosphates (guanine, adenosine, threonine and cytosine) (Amersham Pharmacia, Amersham, UK), 0.1µl of Taq polymerase (Promega UK Ltd, Southampton) and 500nM each of primers *UTR-L1* and *UTR-R1* (from Cuachem Ltd, Glasgow).

UTR-L1 is the forward primer and is complementary to residues -273 to -291 of the 5'UTR of HCV1. It has the sequence:

5'GGA ACT ACT GTC TTC ACG C3'

5

UTR-R1 is the reverse primer and is complementary to residues -6 to -22 of the 5'UTR of HCV1. It has the sequence:

5'ACG GTC TAC GAG ACC TC3'

10

The PCR reaction conditions were as follows: 94°C for 1 minute for 1 cycle; 94°C for 30 seconds; 55°C for 20 seconds; 72°C for 20 seconds for 25 cycles.

1µl of this reaction was then added to 20µl of a second round reaction mix. This mix is the same as the first round reaction mix, except that primers *UTR-L1* and *UTR-L2* are replaced by 500mM of primers *Spec-1* and *UTR-R2*, and 100mM of *UTR-L2*.

15

UTR-L2 is a forward primer which is universal for all HCV genotypes and is complementary to residues -248 to -266 of the 5'UTR of HCV1. It has the following sequence:

20

5'CGT CTA GCC ATG GCG TTA G3'

Spec-1 is a forward primer which is HCV type 1 specific and is complementary to residues -118 to -134 of the 5'UTR of HCV1. It has the following sequence:

25

5'CCI CTC AAT GCC TGG AG3'

UTR-R2 is a reverse primer which is universal for all HCV genotypes and is complementary to residues -50 to -71 of the 5'UTR for HCV1. It has the following sequence:

5 5'GCA GTA CCA CAA GGC CTT TCG C3'

The reaction conditions were as follows: 94°C for 1 minute for 1 cycle; 94°C for 30 seconds; 58°C for 20 seconds; 72°C for 20 seconds for 25 cycles.

10 PCR products were then resolved by agarose gel electrophoresis (NuSieve 3:1, Flowgen Instruments, Lichfield, Staffordshire, UK) run for 40 minutes at 100 Volts in 2% agarose.

15 Samples of known HCV genotypes (determined by RFLP studies) were tested. The results are shown in Figures 1a and 1b. In Figure 1a, the lanes contain (from left to right):

- 1 – molecular weight markers
- 2 – HCV negative control
- 20 3 – HCV 3a
- 4 – HCV 2b
- 5 – HCV 1a
- 6 – HCV 1a
- 7 – negative control
- 25 8 – HCV 1b
- 9 – HCV 1b
- 10 – water control
- 11 – HCV 1b
- 12 – HCV 2b

- 13 – negative control
 - 14 – HCV 1a
 - 15 – HCV 1b
 - 16 – HCV 3a
 - 5 17 – HCV negative control
 - 18 – 1a positive control
 - 19 – water control
 - 20 – molecular weight markers
- 10 In Figure 2a, the lanes contain (from left to right):
- 1 – molecular weight markers
 - 2 – negative control
 - 3 – HCV 1a control
 - 15 4 – HCV 3a control
 - 5 – HCV negative control
 - 6 – HCV 5
 - 7 – negative control
 - 8 – HCV 3a
 - 20 9 – negative control
 - 10 – HCV 2a
 - 11 – HCV 1b
 - 12 – HCV 1a
 - 13 – negative control
 - 25 14 – HCV 3a
 - 15 – HCV 1a
 - 16 – negative control
 - 17 – molecular weight markers

As can be seen, all HCV genotypes result in a 217 base pair band. Genotype 1 HCV generates an additional 88 base pair product.

Example 2

5

RNA was isolated and cDNA produced in the same manner as in Example 1.

Two separate 25µl reaction mixes containing 1µl of cDNA, 3.5mM MgCl₂, 0.2mM each of dATP, dCTP, dGTP and 0.4mM dUTP, 0.25U of AmpliTaq Gold DNA Polymerase (Perkin Elmer), 300nM of *UTR-R2* and 300nM of *L-1* probe were prepared. One contained 300nM of *Spec-1* and the other contained 300nM of *UTR-L2*.

L1 is a HCV genotype 1 specific probe (prepared by Scandinavian Gene Synthesis AB, Koping, Sweden) which is complementary to residues -68 to -93 of the 5'UTR of HCV1 and has the following sequence:

5'FCG CIA CCC AAC ICT ACT IGG CTA GT3'

20 where F=6-FAM, 3'-T+TAMRA

The reaction conditions were as follows: 50°C for 2 minutes, 95°C for 10 minutes; for 1 cycle, 95°C for 15 seconds, 60°C for 60 seconds; for 60 cycles. Fluorescence was detected at 520nm.

25

Samples of known HCV genotypes (determined by RFLP studies) were tested. The results are shown in Figures 2a-f. The following table provides a key to the conditions:

	1	2	3	4	5	6
Primers	(UTR-L2 + UTR-R2)	(Spec-1 + UTR-R2)	(UTR-L2 + UTR-R2)	(Spec-1 + UTR-R2)	(UTR-L2 + UTR-R2)	(Spec-1 + UTR-R2)
A	-ve control	-ve control	sample	Sample	2a	2a
B	-ve control	-ve control	1a	1a	1b	1b
C	-ve control	-ve control	2b	2b	Sample	Sample
D	-ve control	-ve control	3a	3a	sample	Sample
E	-	-	1a	1a	3a	3a
F	1a	1a	5	5	1b	1b
G	sample	sample	1b	1b	3a	3a
H	1b	1b	1a	1a	2b	2b

Thus, it can be seen that HCV-1 can be differentiated from the other subtypes when the primer specific for HCV-1 is used (*Spec-1*) (see Figures 2b, d and f).

5 Similar results are seen in Figures 3a and 3b where the conditions are as follows:

	1 (UTR-L2 + UTR- R2)	2 (UTR-L2 + UTR- R2)	3 (UTR-L2)	4 (UTR-L2)	5 (SPEC-1 + UTR-R2)	6 (SPEC-1 + UTR- R2)	7 (SPEC-1 + UTR- R2)	8 (SPEC-1 + UTR- R2)
A	1a	3a	-ve control	-ve control	1a	3a	-ve control	-ve control

10 This approach relies on sequence specific hybridisation of a fluorescently labelled probe to genotype 1 HCV sequences. These sequences are generated by PCR using at least one primer specific for HCV-1. The probe has a quencher molecule linked to the fluorochrome. As PCR proceeds, the quencher molecule is removed by Taq polymerase from incorporated probes. Fluorescence of the resulting genotype 1 PCR

products can then be detected. The amount of fluorescence generated during the successive rounds of PCR correlates with the amount of PCR product.

Example 3

5

RNA was isolated and cDNA produced in the same manner as in Example 1.

10

Two separate 25µl reaction mixes containing 1µl of cDNA, 3.5mM MgCl₂, 0.2mM each of dATP, dCTP, dGTP and 0.4mM dUTP, 0.1 µl of Taq polymerase stoeffel fragment (Perkin Elmer), 0.1 µl of antiTaq antibody (Gibco BRL – Life Techonolgies, Paisley, Scotland), 60 nM carboxy-x-rhodamine (Cambridge Biosciences, Cambridge, UK), and 300nM of *UTR-R2* were prepared. One contained 300nM of *MBP-LR-ALL* and the other contained 300nM of *MBP-LR-1* (Oswel DNA Service, Southampton).

15

MBP-LR-ALL is a forward primer which is universal for all HCV genotypes and is derived from the primer *UTR-L2*. It has the following sequence:

5' FCA CCT TCA CCC TCA GAA GGM GCG UCT AGC CAT GGC GTT
AG 3'

20

MBP-LR-1 is a forward HCV type 1 specific primer which is derived from the primer *Spec1*. It has the following sequence:

5' FCA CCT TCA CCC TCA GAA GGM GCC GCT CAA TGC CTG GAG 3'

25

(F=FAM; M=MeREDdU and U=Uracil)

The results are shown in Figure 4 where the conditions are as follows:

	1 <i>(MBP-LR-1 + UTR-R2)</i>	2 <i>(MBP-LR-1 + UTR-R2)</i>	3 <i>(MBP-LR-1 + UTR-R2)</i>	4 <i>(MBP-LR-1 + UTR-R2)</i>	5 <i>(MBP-LR-1 + UTR-R2)</i>	6 <i>(MBP-LR-1 + UTR-R2)</i>	7 <i>(MBP-LR-1 + UTR-R2)</i>	8 <i>(MBP-LR-1 + UTR-R2)</i>	9 <i>(MBP-LR-1 + UTR-R2)</i>
A	1a	1a	1a	1a	3a	3a	-ve control	-ve control	-ve control

Claims

1. A method of detecting HCV genotype 1 (HCV-1) in a sample, comprising:
subjecting the sample to an amplification reaction using at least one primer
5 which anneals specifically to the 5' noncoding region (5' NCR) of the HCV-1
genome; and
detecting the product of the amplification reaction.
2. A method as claimed in claim 1, wherein the amplification reaction is the
10 polymerase chain reaction (PCR) or reverse transcriptase polymerase chain reaction
(RT-PCR).
3. A method as claimed in claim 1 or claim 2, further comprising:
detecting whether any HCV genotype is present in the sample by subjecting the
15 sample to an amplification reaction using primers which anneal to a region of the
5'NCR which is conserved between all HCV genotypes; and
detecting the product of this amplification reaction.
4. A method as claimed in claim 3, wherein the primers have the following
20 sequences:
Forward: 5' CGT CTA GCC ATG GCG TTA G 3' (UTR-L2)
Reverse: 5' GCA GTA CCA CAA GGC CTT TCG C 3' (UTR-R2)
5. A method as claimed in any preceding claim, further comprising subjecting the
25 sample to a preliminary amplification reaction to isolate HCV material using primers
universal for all HCV genotypes.
6. A method as claimed in claim 5, wherein the primers comprise the following
sequences:
30 Forward: 5' GGA ACT ACT GTC TTC ACG C 3' (UTR-L1)

Reverse: 5' ACG GTC TAC GAG ACC TC 3' (*UTR-R1*)

7. A method as claimed in any preceding claim, wherein the at least one primer which anneals specifically to the 5' noncoding region (5' NCR) of the HCV-1 genome comprises the sequence:

5' CCI CTC AAT GCC TGG AG 3' (*Spec-1*).

8. A method as claimed in claim 7, wherein the at least one primer which anneals specifically to the 5' noncoding region (5' NCR) of the HCV-1 genome is a forward primer and the reverse primer comprises the sequence:

5' GCA GTA CCA CAA GGC CTT TCG C 3' (*UTR-R2*)

9. A method as claimed in any preceding claim, wherein detection of the product of the or each amplification reaction is by agarose gel electrophoresis.

10. A method as claimed in any one of claims 1 to 8, wherein detection of the product of the or each amplification reaction is by fluorescent analysis in which amplification of HCV-1 specific nucleic acid causes fluorescence of a probe.

11. A method as claimed in claim 10, wherein the probe comprises the sequence:

5' FCG CIA CCC AAC ICT ACT IGG CTA GT 3' (*L1*)

where F=6-FAM, 3'-T+TAMRA.

12. A method as claimed in any one of claims 1 to 6, wherein detection of the product of the or each amplification reaction is by one or more molecular beacon primers.

13. A method as claimed in claim 12, wherein the molecular beacon primer comprises the sequence:

5' FCA CCT TCA CCC TCA GAA GGM GCC GCT CAA TGC CTG GAG 3'

(F=FAM; M=MeREDdU and U=Uracil) (*MBP-LR-1*)

14. A method as claimed in claim 13, wherein the molecular beacon primer is a forward primer and the reverse primer comprises the sequence:

5 5' GCA GTA CCA CAA GGC CTT TCG C 3' (*UTR-R2*)

15. A method as claimed in claim 12, 13 or 14 when appended to claim 2, wherein the primers which anneal to a region of the 5'NCR which is conserved between all HCV genotypes comprise the following sequences:

10

Forward: 5' FCA CCT TCA CCC TCA GAA GGM GCG UCT AGC CAT GGC GTT AG 3' (F=FAM; M=MeREDdU and U=Uracil) *MBP-LR-ALL*

Reverse: 5' GCA GTA CCA CAA GGC CTT TCG C 3' (*UTR-R2*)

15 13. A kit for detecting HCV genotype 1 (HCV-1) in a sample, comprising: at least one primer which anneals specifically to the 5' noncoding region (5' NCR) of the HCV-1 genome.

20 14. A kit as claimed in claim 13, modified by the features of any one of claims 2 to 12.

15. A nucleotide molecule suitable for use in an amplification reaction comprising one of the following sequences:

5' CGT CTA GCC ATG GCG TTA G 3' (*UTR-L2*)

25 5' GCA GTA CCA CAA GGC CTT TCG C 3' (*UTR-R2*)

5' GGA ACT ACT GTC TTC ACG C 3' (*UTR-L1*)

5' ACG GTC TAC GAG ACC TC 3' (*UTR-R1*)

5' CCI CTC AAT GCC TGG AG 3' (*Spec-1*).

5' FCA CCT TCA CCC TCA GAA GGM GCC GCT CAA TGC CTG GAG3'

30 (F=FAM; M=MeREDdU and U=Uracil) (*MBP-LR-1*)

5' FCA CCT TCA CCC TCA GAA GGM GCG UCT AGC CAT GGC GTT
AG 3' (F=FAM; M=MeREDdU and U=Uracil) *MBP-LR-ALL*

15. A pair of primers comprising a nucleotide molecule having the following
5 sequence:

Forward: 5' CGT CTA GCC ATG GCG TTA G 3' (*UTR-L2*)

Reverse: 5' GCA GTA CCA CAA GGC CTT TCG C 3' (*UTR-R2*)

10 Forward: 5' GGA ACT ACT GTC TTC ACG C 3' (*UTR-L1*)

Reverse: 5' ACG GTC TAC GAG ACC TC 3' (*UTR-R1*)

Forward: 5' CCI CTC AAT GCC TGG AG 3' (*Spec-1*).

Reverse: 5' GCA GTA CCA CAA GGC CTT TCG C 3' (*UTR-R2*)

15

Forward: 5'FCA CCT TCA CCC TCA GAA GGM GCC GCT CAA TGC CTG
GAG3' (F=FAM; M=MeREDdU and U=Uracil) (*MBP-LR-1*)

Reverse: 5' GCA GTA CCA CAA GGC CTT TCG C 3' (*UTR-R2*)

20 Forward: 5' FCA CCT TCA CCC TCA GAA GGM GCG UCT AGC CAT GGC
GTT AG 3' (F=FAM; M=MeREDdU and U=Uracil) *MBP-LR-ALL*

Reverse: 5' GCA GTA CCA CAA GGC CTT TCG C 3' (*UTR-R2*)

16. A nucleotide molecule suitable for use as a probe comprising the sequence:

25 5'FCG CIA CCC AAC ICT ACT IGG CTA GT3' (*L1*)

(where F=6-FAM,3'-T+TAMRA).

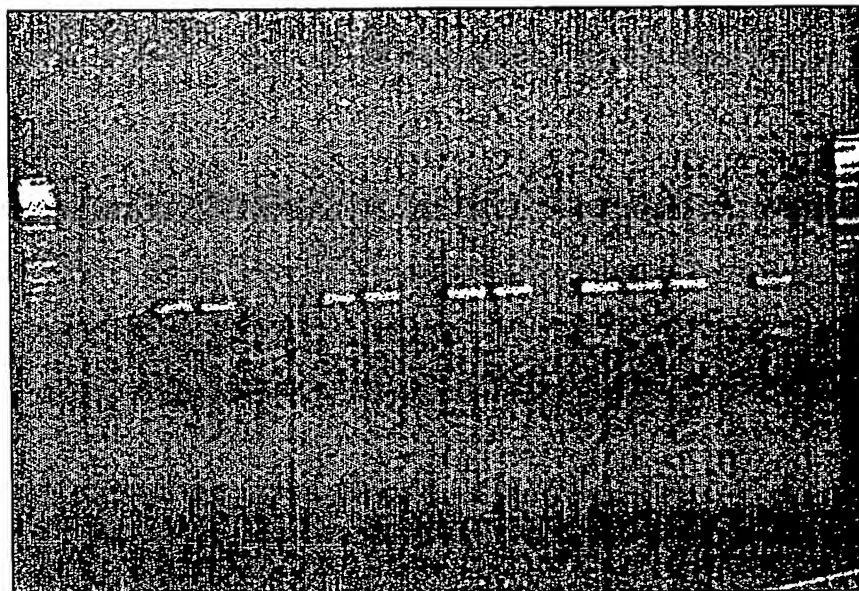
17. A method of detecting HCV genotype 1 (HCV-1) in a sample, comprising:

30 subjecting the sample to an amplification reaction using at least one primer which
anneals to the genome of HCV, a polymerase having a 5'-3' exonuclease activity and an

oligonucleotide probe, which probe anneals specifically to the 5' noncoding region (5' NCR) of the HCV-1 genome and which incorporates a modified nucleotide having a fluorescent characteristic which is modified by one or more neighbouring nucleotides; and

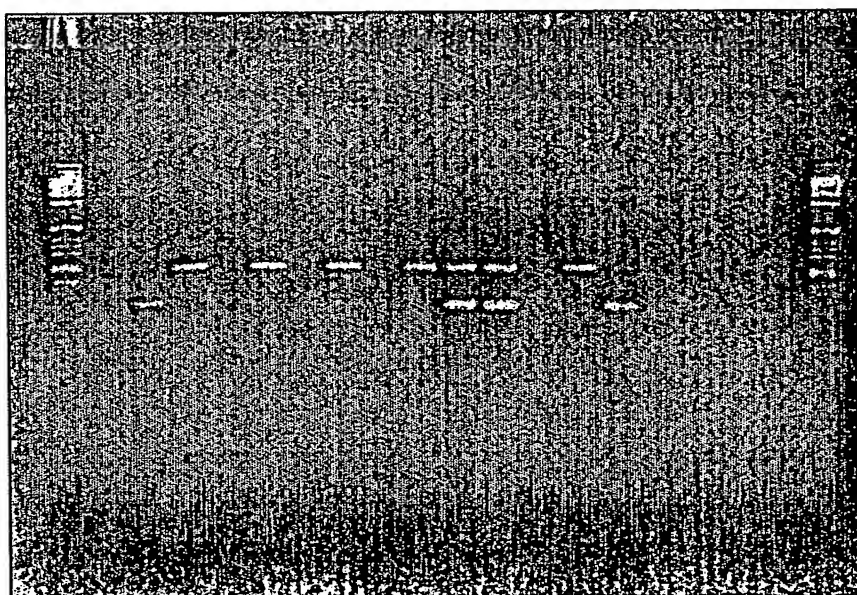
- 5 detecting a change in fluorescence as the oligonucleotide probe is degraded by the exonuclease activity of the polymerase as the polymerase extends the primer and modification of the fluorescent characteristic of the modified nucleotide is reduced.

1 / 11



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

FIG. 1a



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17

FIG. 1b

2 / 11

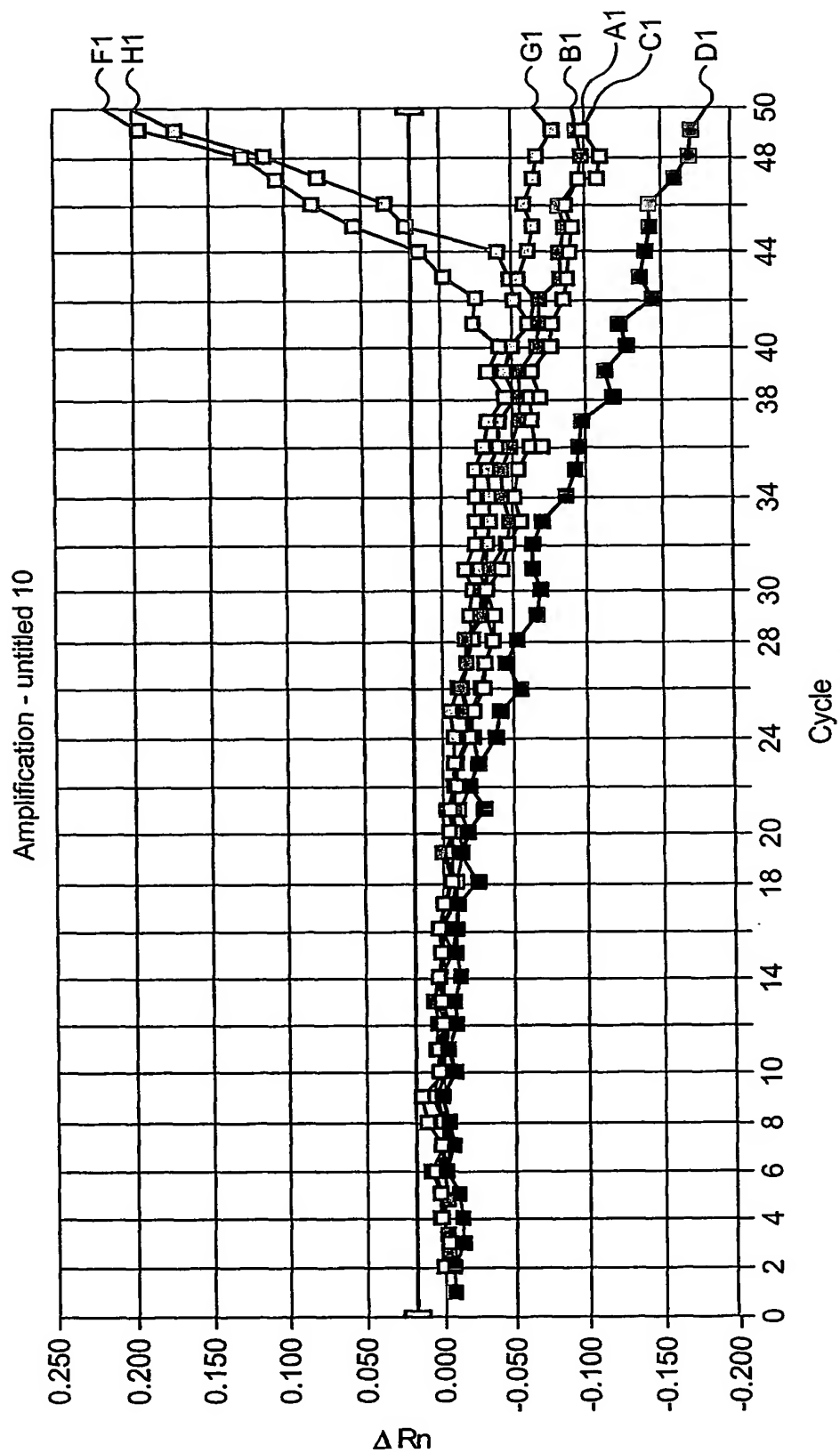


FIG. 2a

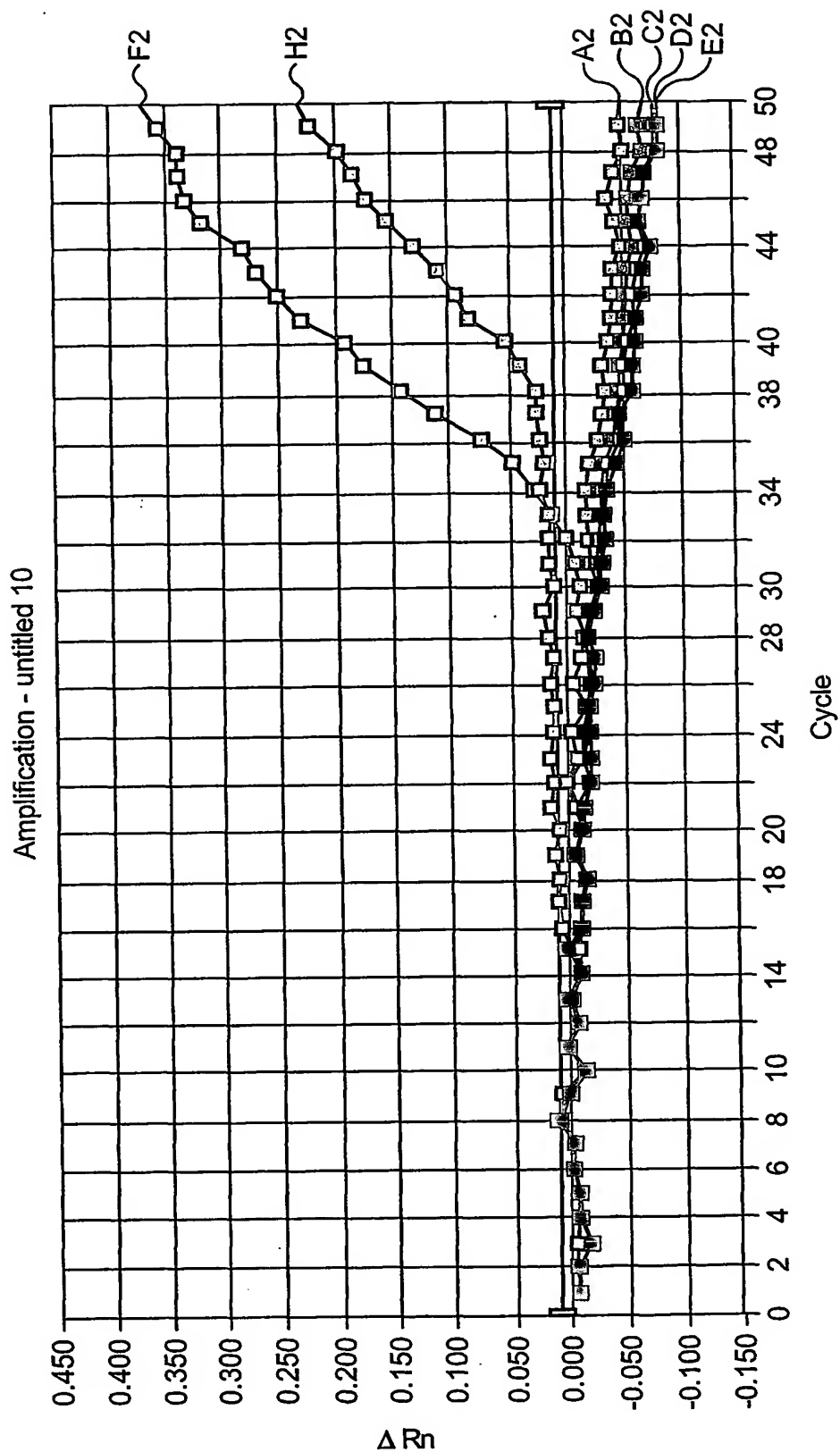


FIG. 2b

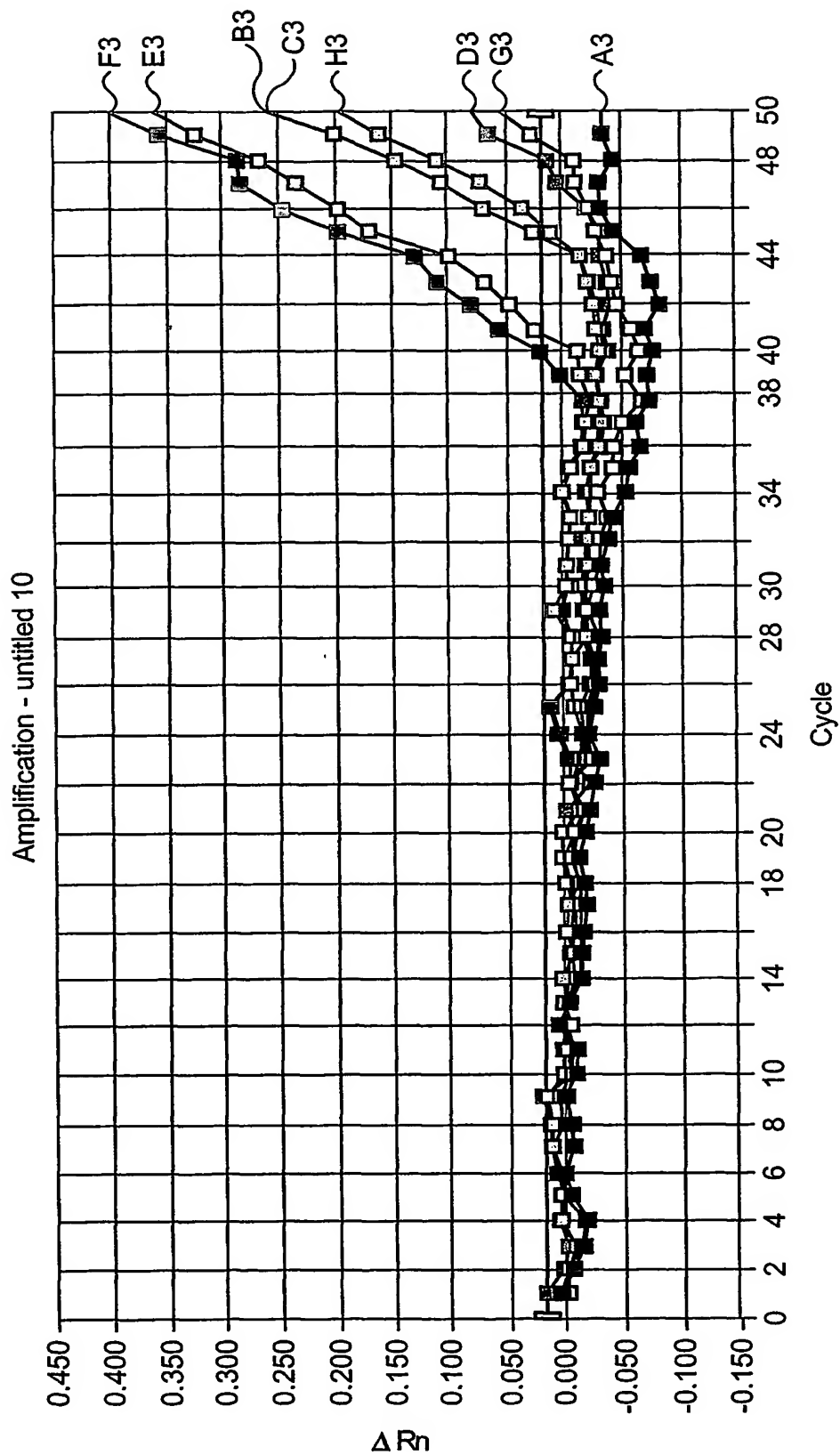


FIG. 2c

5 / 11

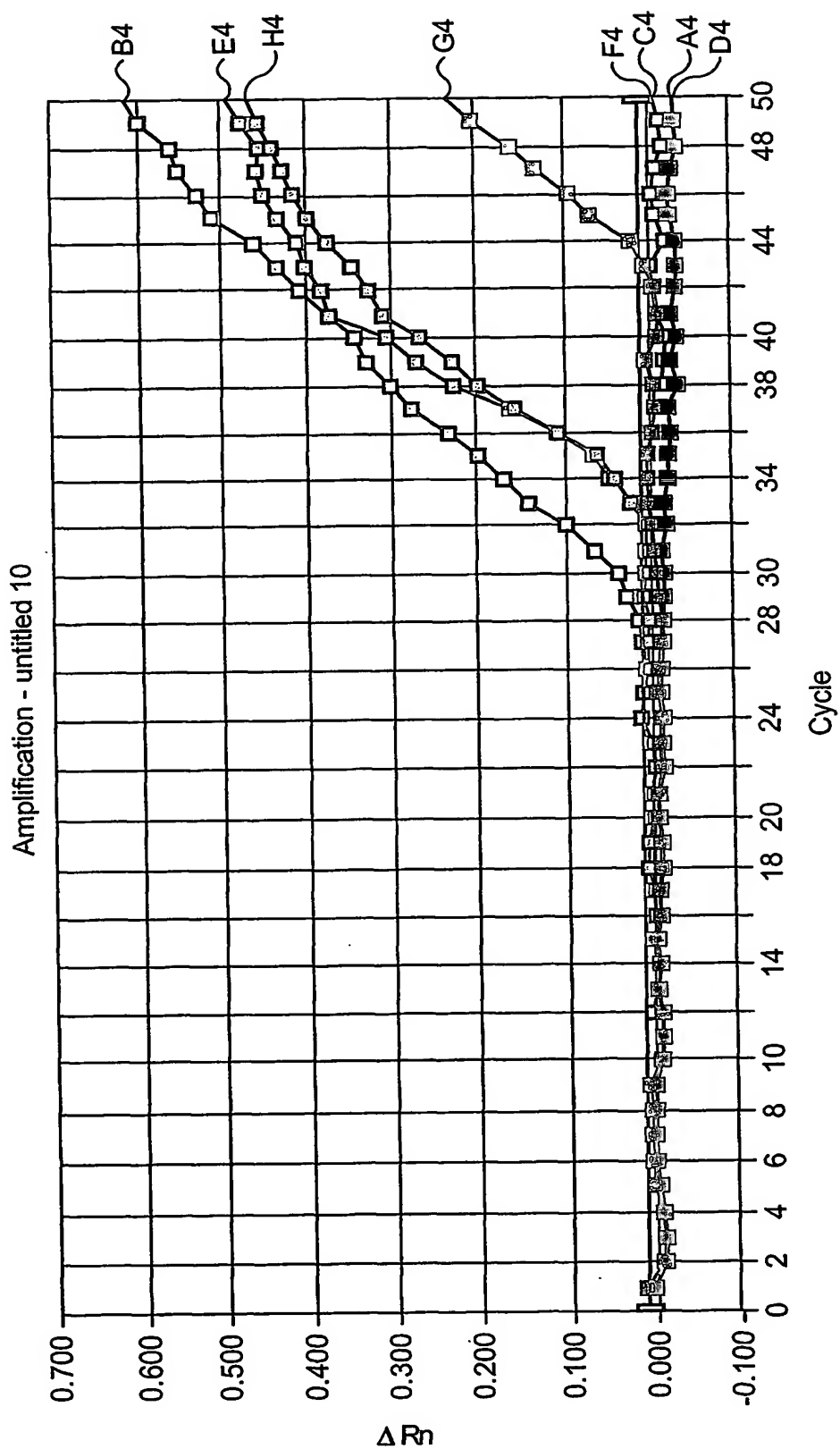


FIG. 2d

6 / 11

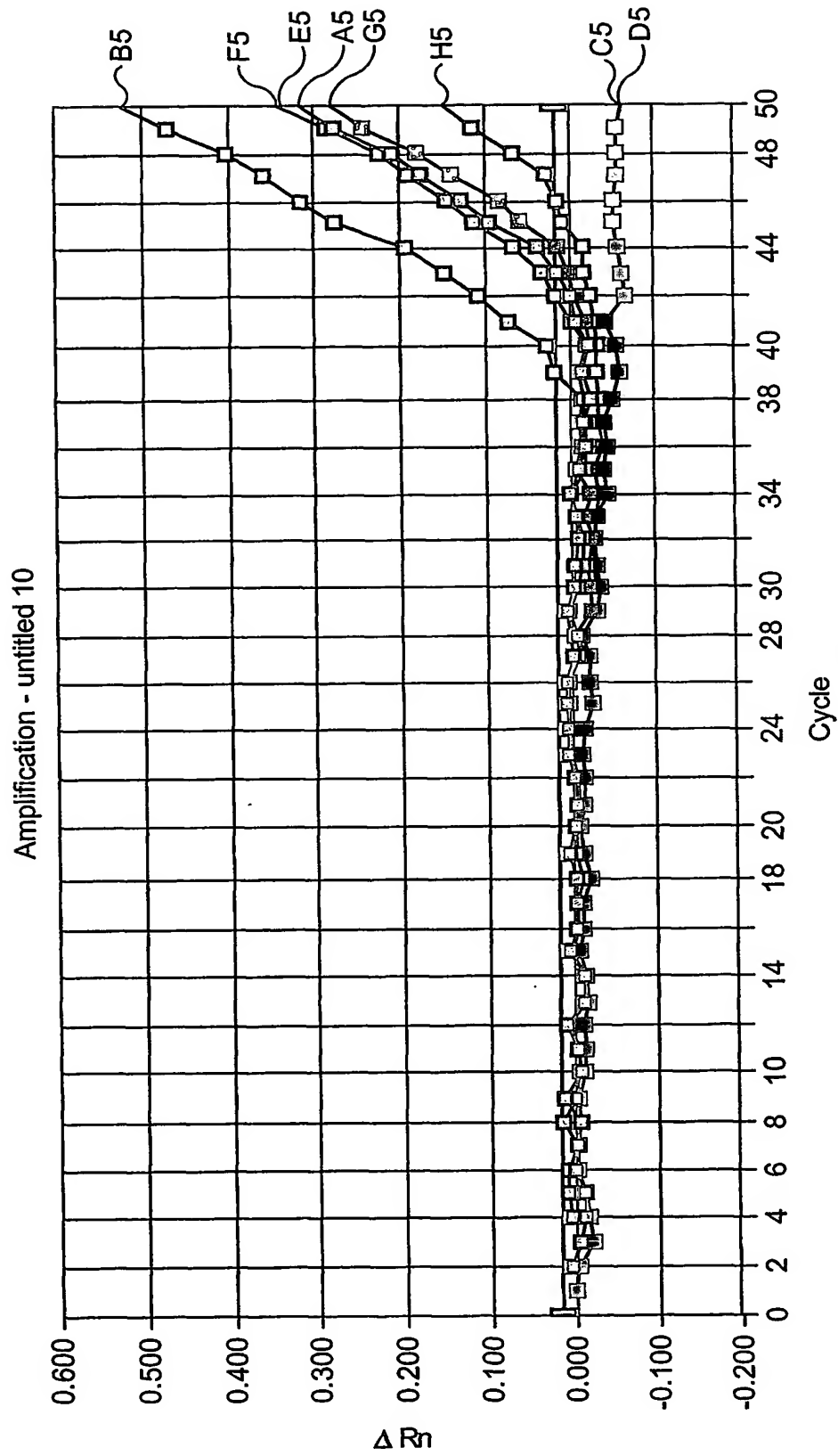


FIG. 2e

7 / 11

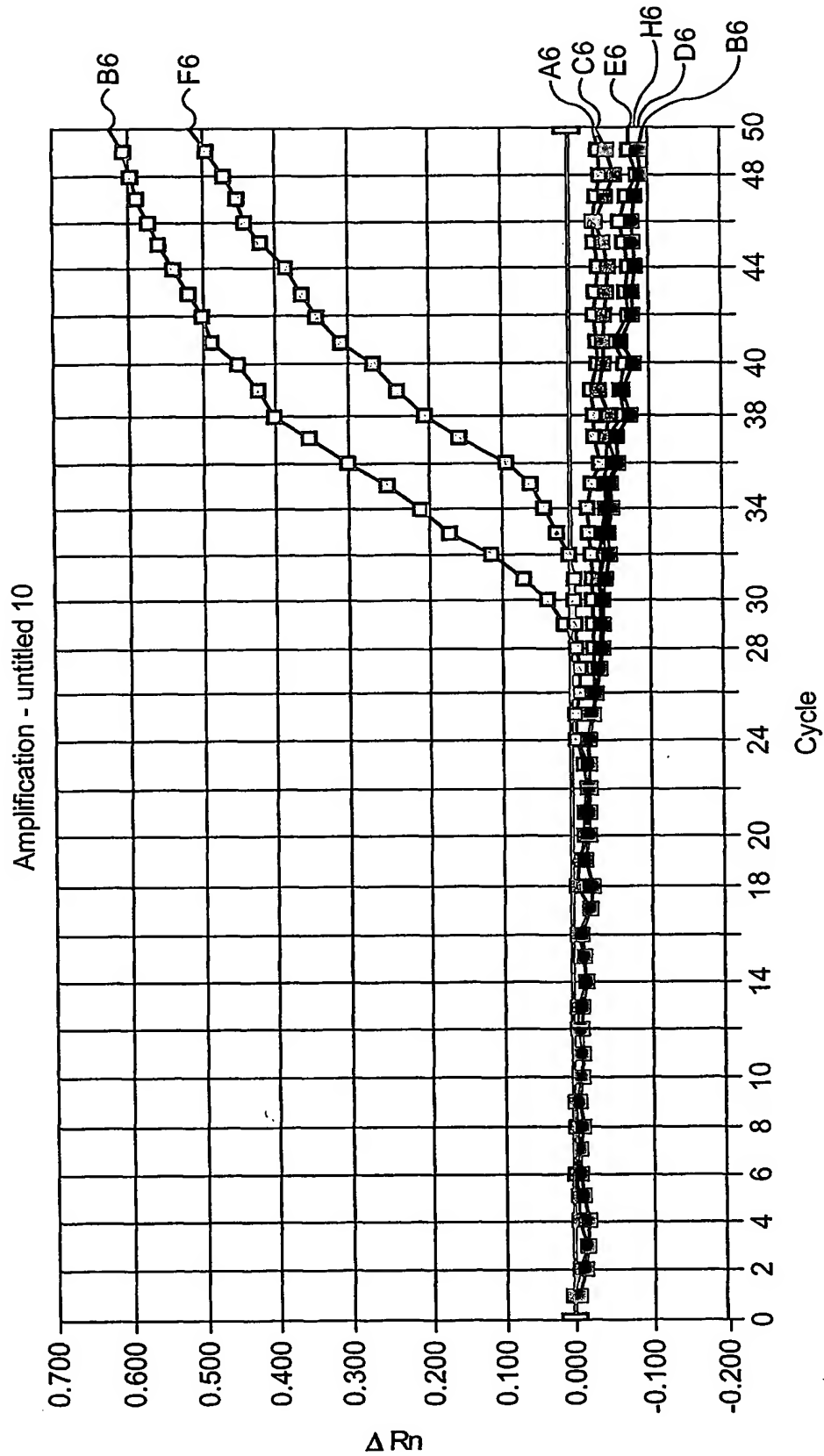


FIG. 2f

8 / 11

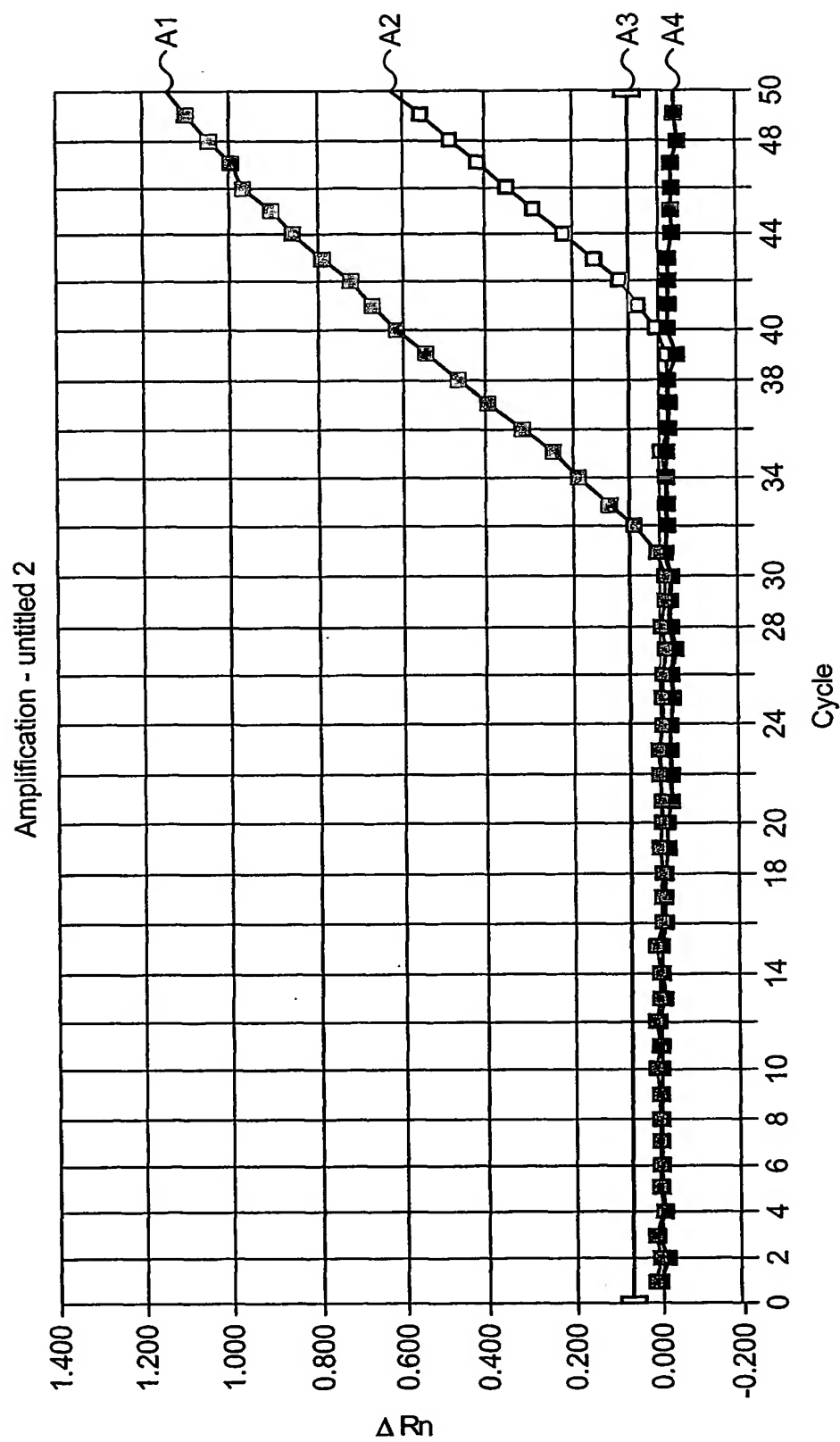


FIG. 3a

9 / 11

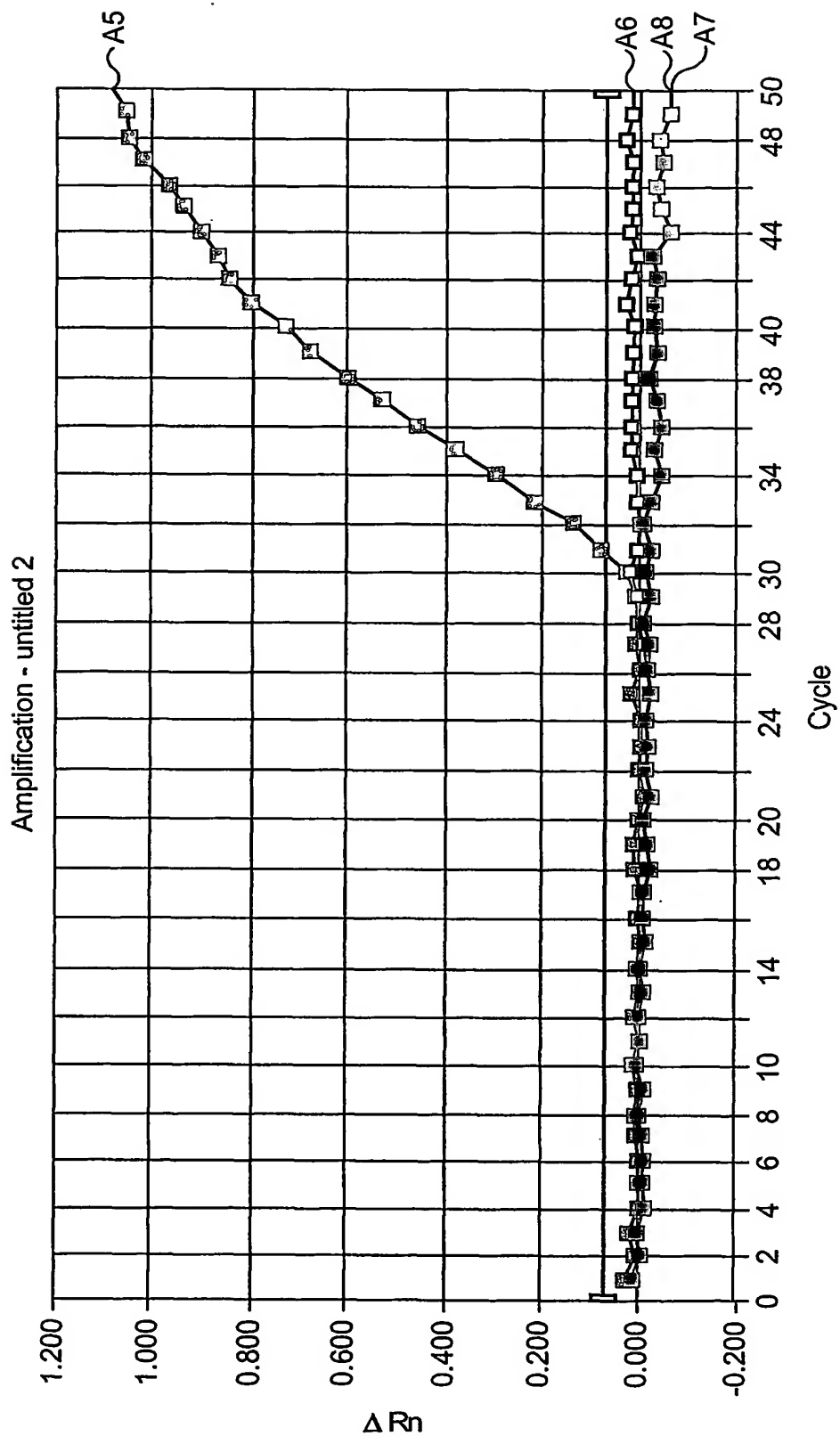


FIG. 3b

10 / 11

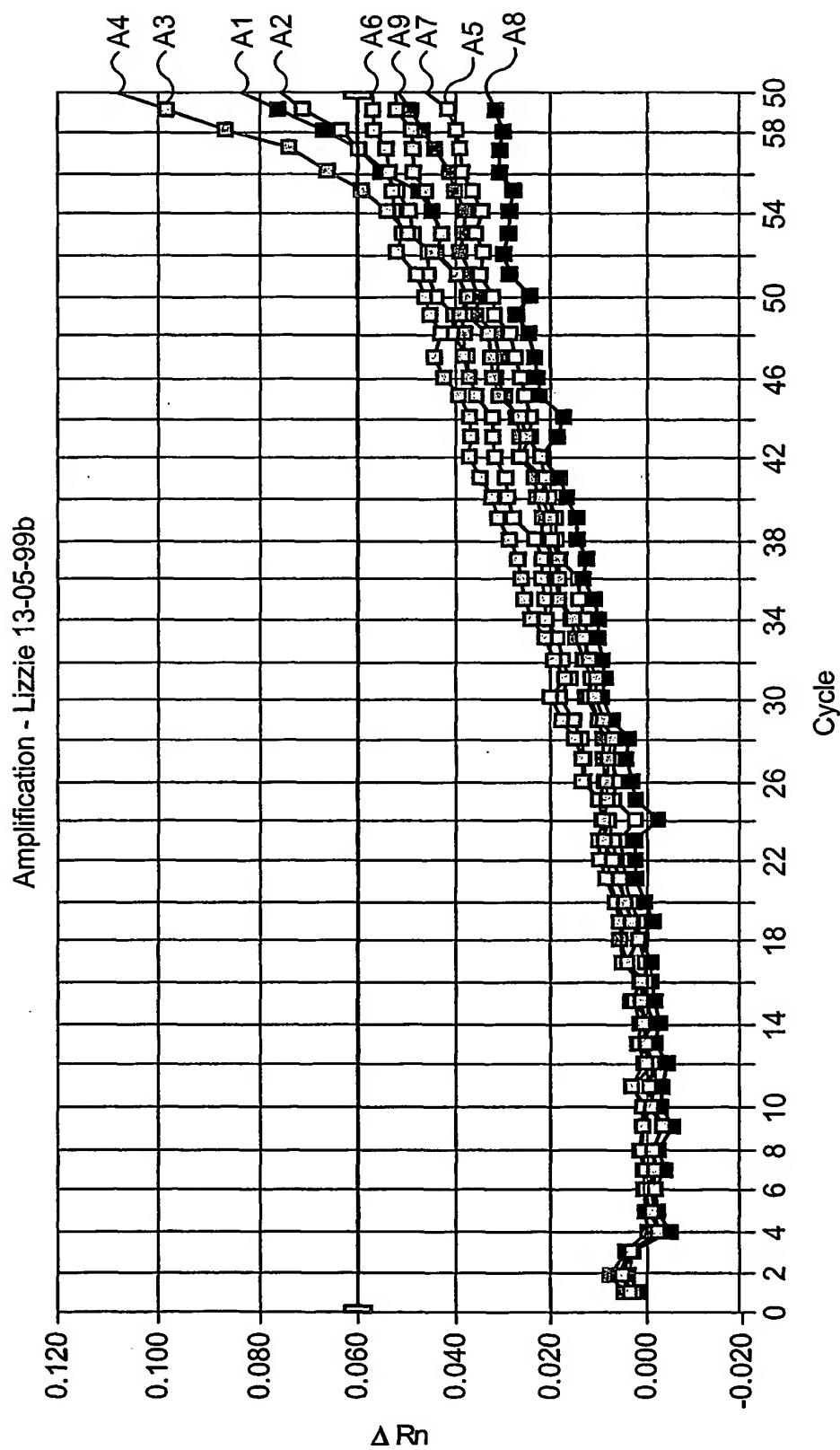


FIG. 4

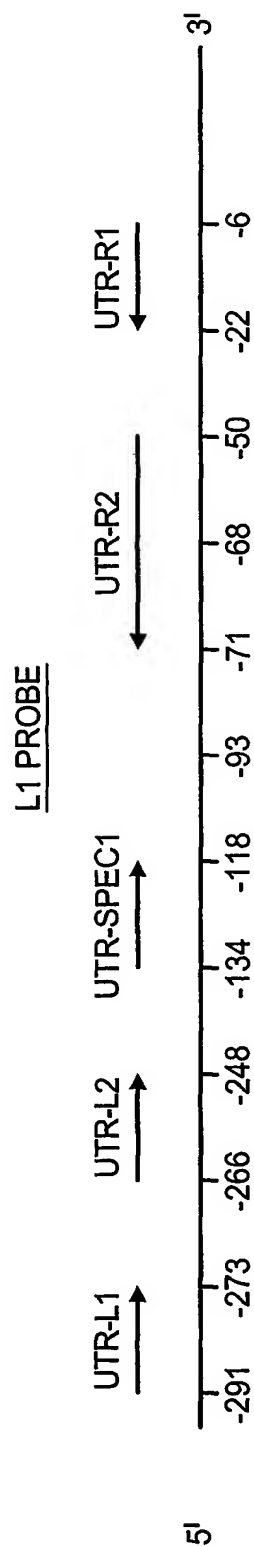


FIG. 5

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
17 July 2003 (17.07.2003)

PCT

(10) International Publication Number
WO 2003/057915 A3

(51) International Patent Classification⁷: C12Q 1/68

(21) International Application Number:
PCT/GB2003/000064

(22) International Filing Date: 10 January 2003 (10.01.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0200526.2 11 January 2002 (11.01.2002) GB

(71) Applicant (for all designated States except US): UNIVERSITY OF SOUTHAMPTON [GB/GB]; Centre for Enterprise and Innovation, Highfield, Southampton SO17 1BJ (GB).

(72) Inventor; and

(75) Inventor/Applicant (for US only): ROSENBERG, William, Malcolm, Charles [GB/GB]; Centre for Enterprise and Innovation, Highfield, Southampton SO17 1BJ (GB).

(74) Agents: LEE, Nicholas, John et al.; Kilburn & Strode, 20 Red Lion Street, London WC1R 4PJ (GB).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(88) Date of publication of the international search report:
8 January 2004

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS OF DETECTING HCV GENOTYPE 1 (HCV-1) BY USING PRIMERS SPECIFIC FOR THE 5' NON-CODING REGION (NCR) OF THE HCV GENOME

(57) Abstract: The present invention provides a method of detecting HCV genotype 1 (HCV-1) in a sample which is based on the finding that the 5' non-coding region (NCR) of the HCV genome is conserved between HCV-1 quasi-species but not between other HCV subgroups. The method comprises subjecting the sample to an amplification reaction using at least one primer which anneals specifically to the 5' noncoding region (5' NCR) of the HCV-1 genome, and detecting the product of the amplification reaction. Kits and primers suitable for carrying out the method are also provided.



WO 2003/057915 A3

INTERNATIONAL SEARCH REPORT

Intern: Application No

PCT/GB 00064

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, MEDLINE, EMBASE, BIOSIS, SEQUENCE SEARCH, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 46469 A (KINOSHITA MORITOSHI ; ISHIDA YUSUKE (JP); KUBOKI MAKOTO (JP); OTSUK) 28 June 2001 (2001-06-28) abstract	1-3
X	US 5 851 759 A (WEINER AMY J) 22 December 1998 (1998-12-22) column 1 -column 2 column 8 -column 12	1-3, 5, 9, 10, 12, 16, 17, 21
Y		4, 6-8, 11, 13-15, 18-20



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

28 October 2003

Date of mailing of the international search report

05/11/2003

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

BROCHADO GARGANTA, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BUKH J ET AL: "SEQUENCE ANALYSIS OF THE 5' NONCODING REGION OF HEPATITIS C VIRUS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 89, no. 11, 1 June 1992 (1992-06-01), pages 4942-4946, XP000384404 ISSN: 0027-8424 page 4942 page 4945 -page 4946 figure 1	4,6-8, 11, 13-15, 18-20
A	--- TANAKA Y ET AL: "Selective Binding of Hepatitis C Virus Core Protein to Synthetic Oligonucleotides Corresponding to the 5' Untranslated Region of the Viral Genome" VIROLOGY, ACADEMIC PRESS, ORLANDO, US, vol. 270, no. 1, 25 April 2000 (2000-04-25), pages 229-236, XP004436062 ISSN: 0042-6822 the whole document	1-21
A	--- DI BISCEGLIE A M: "Hepatitis C" LANCET, XX, XX, vol. 351, no. 9099, 31 January 1998 (1998-01-31), pages 351-355, XP004265097 ISSN: 0140-6736 the whole document -----	1-21

INTERNATIONAL SEARCH REPORT

Inte..... application No.
PCT/GB 03/00064

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

The last 6 claims of the application have been renumbered (now, claims 16-21),
as the numbering was wrong and several claims had the same number.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Internat
PCT/GB 00064

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0146469	A	28-06-2001	WO 0146469 A1	28-06-2001
			JP 2001238687 A	04-09-2001
US 5851759	A	22-12-1998	CA 2251673 A1	30-10-1997
			EP 0904406 A1	31-03-1999
			JP 2000508907 T	18-07-2000
			WO 9740190 A1	30-10-1997

This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.